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Quantification of anisotropy and orientation in 3D electron microscopy and diffusion tensor imaging in injured rat brain

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ABSTRACT

Diffusion tensor imaging (DTI) reveals microstructural features of grey and white matter non-invasively. The contrast produced by DTI, however, is not fully understood and requires further validation. We used serial block-face scanning electron microscopy (SBEM) to acquire tissue metrics, i.e., anisotropy and orientation, using three-dimensional Fourier transform-based (3D-FT) analysis, to correlate with fractional anisotropy and orientation in DTI. SBEM produces high-resolution 3D data at the mesoscopic scale with good contrast of cellular membranes. We analysed selected samples from cingulum, corpus callosum, and perilesional cortex of sham-operated and traumatic brain injury (TBI) rats. Principal orientations produced by DTI and 3D-FT in all samples were in good agreement. Anisotropy values showed similar patterns of change in corresponding DTI and 3D-FT parameters in sham-operated and TBI rats. While DTI and 3D-FT anisotropy values were similar in grey matter, 3D-FT anisotropy values were consistently lower than fractional anisotropy values from DTI in white matter. We also evaluated the effect of resolution in 3D-FT analysis. Despite small angular differences in grey matter samples, lower resolution datasets provided reliable results, allowing for analysis of larger fields of view. Overall, 3D SBEM allows for more sophisticated validation studies of diffusion imaging contrast from a tissue microstructural perspective.

Introduction

Despite the wide use of diffusion tensor imaging (DTI) in clinics and research, the biological interpretation of the contrast produced by DTI is lacking. Among all of the magnetic resonance imaging (MRI) techniques, DTI is the method of choice for non-invasively obtaining microstructural information from brain tissue. DTI probes the tissue microstructure by measuring the diffusion of water, i.e., the random motion of water molecules, restricted by tissue microstructures (Basser et al., 1994; Basser and Pierpaoli, 1996; Le Bihan, 2003). A typical voxel in DTI, e.g., $100 \times 100 \times 100\,\mu m^3$ in rodent brain or $2 \times 2 \times 2\,mm^3$ in humans, is highly heterogeneous, however, and contains a variety of cellular structures whose contribution to the restricted diffusion of the water molecules is only partially understood.

Histological characterization, mainly using light microscopy, has predominantly been used to interpret the DTI quantitative parameters in terms of the underlying cellular structures in normal and pathological brain (Dauguet et al., 2007; Leergaard et al., 2010; Wang et al., 2014; Aggarwal et al., 2015; Sierra et al., 2015). Although the combination of DTI and conventional histology provides insights into the DTI contrast, light microscopy has several major restrictions in terms of interpreting the imaging contrast, such as inability to produce three-dimensional (3D) images, staining specificity, and spatial resolution. Recent advances have made 3D microscopic data available for correlation studies, such as confocal light microscopy (Jespersen et al., 2012; Khan et al., 2015; Schilling et al., 2016), polarised light microscopy (Axer et al., 2011, 2016), serial optical coherence scanning (Wang et al., 2014, 2015), or whole-brain multiphoton imaging in combination with clearing tissue techniques (Chang et al., 2017a,b). The main physical barriers for water molecule motion are the cellular membranes. The resolution of light microscopy in the techniques mentioned above, however, cannot reliably resolve individual membranes. Even with the use of lipophilic carbocyanine dyes (e.g., DiI), which label cellular membranes (Chazotte, 2011), only "macrostructural information" of the cellular barriers for

Abbreviations: 3D-FT, three dimensional Fourier transform-based analysis; AI, anisotropy index; DEC, directionally encoded coloured map; DTI, diffusion tensor imaging; EM, electron microscopy; FA, fractional anisotropy; HM, high magnification; LM, low magnification; MRI, magnetic resonance imaging; SBEM, serial block-face scanning electron microscopy; TBI, traumatic brain injury; V1, principal eigenvector; VOI, volume-of-interest.

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water molecules can be determined (Budde et al., 2011; Khan et al., 2015). Another limitation of the histochemistry and immunohistochemistry staining methods used for light microscopy is that the dyes and antibodies can be used to trace only a limited number of cellular components within the same preparation.

Electron microscopy (EM) provides the contrast and high resolution required for visualizing cellular membranes, but has not reached a large enough spatial scale to cover the scale of the MRI voxel. Serial block-face scanning electron microscopy (SBEM) produces 3D datasets in high-resolution at the mesoscopic scale (on the order of a few hundred micrometres) (Denk and Horstmann, 2004). Imaging of the block-face in SBEM is performed by scanning electron microscopy (SEM) using back-scattered electron detection and low beam energies (Denk and Horstmann, 2004; Helmstaedter et al., 2008). An ultra-microtome inside a vacuum chamber repeatedly removes the top section of a block-face using a diamond knife. In each round, the newly exposed block-face is imaged. As the enhanced tissue staining protocol for EM allows for visualisation of all the membranes, SBEM is an ideal method for obtaining insight into the 3D tissue microstructural environment with a large field of view that is comparable to the voxel size of MRI.

Fourier transform-based (FT) analysis extracts information about the organization and orientation of structures of an image (Josso et al., 2015; Marquez, 2006). In a photomicrograph, FT analysis uses the intensity patterns of the stained cellular structures to measure their anisotropy and orientation. As the FT method is intrinsically different from DTI, anisotropy values from FT analysis might differ from DTI fractional anisotropy (FA) values, but these two measures are expected to correlate. Previous FT analysis work studied microstructural changes in histological photomicrographs of normal and pathological brain tissue (Budde et al., 2011; Salo et al., 2017). These studies demonstrated that FT analysis offers a direct and quantitative assessment of the anisotropy and orientation of the microstructures within a tissue, allowing investigators to obtain insight into the source of the DTI contrast.

In this study, we exploited the potential of SBEM to provide 3D high-resolution images of diffusion-restricting structures in tissue. To obtain quantitative metrics comparable to DTI data, we extended the existing 2D Fourier analysis to 3D Fourier transform-based analysis (3D-FT). We calculated the anisotropy and orientation in the SBEM volumes of the cingulum, corpus callosum, and perilesional cortex in a sham-operated rat and rats with severe traumatic brain injury (TBI). Finally, we compared the anisotropy and orientation data obtained from SBEM to the fractional anisotropy (FA) and orientation data obtained from DTI of the same samples. This method may provide novel insights into the microstructural environment probed by water molecules in diffusion MRI.

Materials and methods

Animals

Three adult male Sprague-Dawley rats (10-weeks old, weight 300–350 g, Harlan Netherlands B.V., Horst, Netherlands) were used in the study. The animals were housed in a room (22 ± 1 °C, 50%–60% humidity) with 12 h light/dark cycle and free access to food and water. All animal procedures were approved by the Animal Care and Use Committee of the Provincial Government of Southern Finland and performed according to the guidelines set by the European Community Council Directives 86/609/EEC.

Traumatic brain injury model

TBI was induced by lateral fluid percussion injury (Kharatishvili et al., 2006). Rats were anesthetised with a single intraperitoneal injection (6 ml/kg) of a mixture of sodium pentobarbital (58 mg/kg), magnesium sulphate (127.2 mg/kg), propylene glycol (42.8%), and absolute ethanol (11.6%). A craniotomy (⌀ 5 mm) was performed between bregma and lambda on the left convexity (anterior edge 2.0 mm posterior to bregma; lateral edge adjacent to the left lateral ridge). Lateral fluid percussion injury was induced by a transient fluid pulse impact (21–23 ms) against the exposed intact dura using a fluid-percussion device. The impact pressure was adjusted to 3.2–3.4 atm to induce a severe injury. Sham-operation included all the surgical procedures except the impact.

Tissue processing

EM studies are challenging because of the labour-intensive sample preparation, which limits the number of samples that can be analysed. For this proof-of-concept study, we prepared a total of 12 samples, from ipsi- and contralateral cingulum/corpus callosum and perilesional cortex of one sham-operated and two TBI rats (TBI #1 and TBI #2). The three rat brains and the samples were prepared according to a carefully planned protocol including both DTI and SBEM to preserve the ultrastructure of the tissue.

Five months after TBI or sham operation, the rats were transcardially perfused using 0.9% NaCl (30 ml/min) for 2 min followed by 4% paraformaldehyde (PFA; 30 ml/min) at 4 °C for 25 min. The brains were removed from the skull and post-fixed in 4% PFA/1% glutaraldehyde overnight at 4 °C. The brains were placed in 0.9% NaCl for at least 12 h to remove excess PFA.

Ex vivo DTI and data processing

The rat brains were scanned *ex vivo* in a vertical 9.4 T/89 mm magnet (Oxford Instruments PLC, Abingdon, UK) interfaced with a DirectDrive console (Varian Inc., Palo Alto, CA, USA) using a quadrature volume RF-coil (Ø = 20 mm; Rapid Biomedical GmbH, Rimpar, Germany) as both transmitter and receiver. During imaging, the brains were immersed in perfluoropolyether (Solexis Galden®, Solvay, Houston, TX, USA) to avoid signals from the surrounding area.

The data were acquired using a 3D segmented spin-echo EPI sequence (TR = 1000 ms, TE = 35 ms, data matrix $128 \times 96 \times 96$, FOV $19.2 \times 14.4 \times 14.4$ mm^3, resolution $0.150 \times 0.150 \times 0.150$ mm^3) with four segments. The acquisition comprised a total of 129 vol, with 3 sets of 42 uniformly distributed directions with diffusion weighting (b-values = 2000, 3000, and 4000 s/mm^2, $\delta = 6$ ms, $\Delta = 11.5$ ms) and three images without diffusion weighting. The total scan time was 14 h, 24 min, and 27 s. The samples were imaged at room temperature, and the temperature variation of the sample over the DTI measurement period as measured in separate experiments under identical conditions was less than ±0.25 °C (Laitinen et al., 2010).

The DTI data were first converted to NIfTI format, and pre-processing and analyses were performed using the FMRIB Software Library (FSL 5.0, http://fsl.fmrib.ox.ac.uk/fsl/fslwiki). Pre-processing steps consisted of brain extraction using the FSL Brain Extraction Tool, followed by simultaneous motion correction and eddy current correction using the FSL eddy tool. The analysis was performed in a single DTIFIT run with all the different b-values specified simultaneously using ordinary least-squares fitting. The maps acquired were FA, the principal eigenvector (V1), the second eigenvector (V2), the third eigenvector (V3), the first eigenvalue (L1), the second eigenvalue (L2), the third eigenvalue (L3), and directionally-encoded fractional anisotropy map (DEC-FA) (Fig. 1A).

Tissue preparation for SBEM

After the *ex vivo* DTI, the brains were placed in 0.9% NaCl for at least 4 h to remove excess perfluoropolyether, and were then sectioned into 1-mm thick coronal sections with a vibrating blade microtome (VT1000s, Leica Instruments, Germany). Sections from −3.80 mm from bregma from each brain were selected and further dissected into smaller samples containing the areas of interest. We collected four samples from each brain: ipsilateral and contralateral perilesional cortex, and ipsilateral and contralateral cingulum/corpus callosum (Fig. 1B). Note that the dissected areas were bigger than that required for imaging to ensure proper co-